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# Mass spectrometric detection and measurement of $N^2$-(2′-deoxyguanosin-8-yl)PhIP adducts in DNA

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**Abstract**

Capillary column gas chromatography–electron-capture mass spectrometry (GC–MS) and microbore liquid chromatography–positive ion electrospray mass spectrometry (LC–MS) have been used to measure the carcinogenic, food-derived, heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) adducted at C-8 of deoxyguanosine in DNA. For GC–MS analysis, PhIP was released from adducted DNA by alkaline hydrolysis and analysed as the di(3,5-bistrifluoromethylbenzyl) derivative, while for LC–MS analysis, the nucleoside $N^2$-(2′-deoxyguanosin-8-yl)PhIP was generated by enzymic digestion of DNA and analysed intact. A deuterated analogue of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was used as an internal standard in both assays, which each had a limit of quantification of 200 pg/500 μg DNA. The two methods were used to analyse DNA extracted from h1A2v2 cells and HCT116 cells that had been exposed to PhIP. 

*Keywords:* DNA; 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

## 1. Introduction

The food-derived heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is formed when meat is cooked [1] and is the most abundant of the heterocyclic mutagenic amines generated when red meat such as beef is fried at high temperatures [2]. PhIP is a potent mammalian cell mutagen [3] and has been shown to be procarcinogenic in rodent bioassays [4,5]. The daily and lifelong exposure of humans to this carcinogen via food, albeit at parts per billion levels, has prompted speculation regarding its role in the aetiology of diet associated human cancer. Because of the large interindividual differences in metabolism in the human population, measuring exposure to a carcinogen does not necessarily reflect carcinogenic risk. However, DNA adducts are the chemical endpoint of metabolic activation of PhIP and so reflect the biologically active dose to which cells are exposed. PhIP, after metabolic activation, has been shown to react with DNA, both nuclear and mitochondrial [6], particularly at the C-8 position of deoxyguanosine residues [7–9]. It is generally accepted that it is these DNA adducts which give rise to mutations induced by misrepair of damaged DNA or as a consequence of errors during replication. If

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measurable, such adducts could be very useful biomarkers of PhIP-induced DNA damage.

Traditionally, DNA adduct levels have been identified and semi-quantified using techniques such as $^{32}P$ postlabelling and, where high doses have been administered, by HPLC with radiometric detection. Mass spectrometry can offer equivalent or better sensitivity and much greater specificity, however, and Friesen and colleagues have measured PhIP–DNA adducts by alkaline hydrolysis of DNA from animal and human tissues [10,11], with the liberated PhIP being quantified by selected-ion monitoring gas chromatography–mass spectrometry (GC–MS). Accelerator mass spectrometry has also been used to quantify $^{14}C$ recovered from $^{14}C$ PhIP-adducted DNA obtained from laboratory animals [12] and human subjects [13] treated with doses of PhIP comparable to those consumed in a cooked meat meal. Both of these methods provide a specific measurement of PhIP adducted to DNA but do not identify which nucleosides are involved. We have investigated methods based on GC–MS and liquid chromatography–mass spectrometry (LC–MS) to detect and measure PhIP adducted at C-8 of deoxyguanosine [$N^2$-(2′-deoxyguanosin-8-yl)PhIP] in DNA. Direct analysis of nucleosides adducted to PhIP by GC–MS is difficult to achieve since the physicochemical properties of these molecules make them unsuitable for gas chromatography. We have therefore examined indirect approaches based on cleavage of PhIP from the adducted nucleoside or DNA. The liberated amine can then be measured using a GC–MS method that has previously been developed in our laboratory [14]. The possibility of directly analysing the intact nucleoside–PhIP adduct by LC–MS has also been explored. In order to ensure accurate quantification of the PhIP adducts, a deuterated analogue of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was synthesised for use as an internal standard in these assays.

## 2. Experimental

### 2.1. Chemicals

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 1-methyl-2-nitro-6-phenylimidazo[4,5-*b*]pyridine (2-nitro-PhIP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-2-$^{14}C$ ([$^{14}C$]PhIP) and 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]-pyridine ([$^2H_3$]PhIP) were purchased from Toronto Research Chemicals (Toronto, Canada). 3,5-Bistrifluoromethylbenzyl bromide, diisopropylethylamine and dodecane were obtained from Sigma–Aldrich (Poole, UK). Other organic solvents and hydrochloric acid were of ''Analar'' grade. Diisopropylethylamine was passed through a column of basic alumina, and ethyl acetate was redistilled before use. Thin-layer chromatography (TLC) plates were precoated with silica gel 60 $F_{254}$ and were obtained from Merck (Poole, UK). Bond-Elut columns ($C_{18}$, 200 mg, 3 ml) were obtained from Anachem (Luton, UK). Reacti-vials were supplied by Pierce and Warriner (Chester, UK). Analytical high-performance liquid chromatography (HPLC) was performed with a HiChrom (Theale, UK) RPB base deactivated column (25 cm×4.6 mm I.D.). All other chemicals used were of analytical grade or better and were obtained from Sigma (Poole, UK). *N*-(Deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine [$N^2$-(2′-deoxyguanosin-8-yl)PhIP] was synthesised from PhIP by the method of Lin et al. [8] while the deuterated analogue, $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP was synthesised from [$^2H_3$]PhIP as described below (Fig. 1). Where possible, all synthetic procedures were carried out in reduced light.

### 2.2. Synthesis

#### 2.2.1. 2-Nitro-[$^2H_3$]PhIP

[$^2H_3$]PhIP, 15 mg, was dissolved in aqueous acetic acid (1:1, v/v, 1.5 ml) which was added slowly to sodium nitrite solution (0.5 ml, 6 *M*) at 0–4°C (on ice) for 45 min. The reaction was terminated by the addition of 30 ml deionised water. The reaction mixture was applied to pre-conditioned $C_{18}$ Bond-Elut columns that were then washed to neutral pH with water. Each column was eluted with 1 ml methanol, the fractions pooled and dried under a stream of nitrogen. After reconstitution in a minimal volume of chloroform, the product was applied to a silica gel column (24 cm×1.3 cm) and eluted with methanol (100 ml), chloroform–methanol (9:1, v/v, 100 ml) and chloroform (100 ml). Deuterium-la-

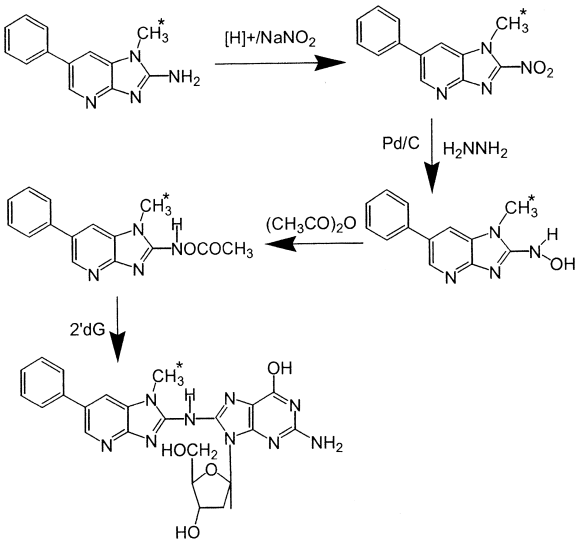


Fig. 1. Synthesis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP (* indicates position of deuterium label).

belled 2-nitro-PhIP eluted in the first fraction and its identity and purity were confirmed by TLC (chloroform–methanol, 10:1, v/v) and HPLC with reference to an authentic sample of 2-nitro-PhIP. The product was air dried, reconstituted in methanol and stored at −20°C.

### 2.2.2. $N^2$-(2′-Deoxyguanosin-8-yl)-[$^2H_3$]PhIP

2-Nitro-[$^2H_3$]PhIP (4 μmol) was dissolved in nitrogen purged tetrahydrofuran (2 ml) to which 10% palladium–charcoal catalyst (2 mg) was added and the mixture kept on ice with stirring. Hydrazine hydrate (10 μl) was added and the mixture stirred for 40 min. The formation of deuterium-labelled *N*-hydroxy PhIP was assessed by TLC (chloroform–methanol, 9:1, v/v) and HPLC and, when complete, the reaction was terminated by the addition of 10 volumes of cold, nitrogen-purged ethylenediaminetetraacetic acid (EDTA) buffer (10 m*M*, pH 4.6). The catalyst was removed by centrifugation and the reaction mixture applied to a $C_{18}$ Bond-Elut column maintained at <4°C and protected from light. The column was washed with two bed volumes of EDTA buffer, then *N*-hydroxy PhIP was eluted with one bed volume of dimethylsulfoxide (DMSO)–methanol (4:1, v/v). To this solution, acetic anhydride was added (47 μl over 5 min with continual stirring on ice) and the acetylation reaction allowed to proceed for 10 min. The acetoxy-PhIP product was then added to 2′-deoxyguanosine solution (5 m*M* in 10 ml potassium citrate buffer, 10 m*M*, pH 5.0 containing 1 m*M* EDTA) and mixed at 37°C for 30 min. Deuterated $N^2$-(2′-deoxyguanosin-8-yl)PhIP separated as a precipitate and the yield was maximised by refrigeration (4°C, 8 h). The precipitated adduct was recovered by centrifugation, dried under vacuum, reconstituted in a minimal volume of dimethylformamide (DMF)–chloroform (1:4, v/v) and purified by silica gel column chromatography by washing with 10 ml DMF–chloroform (1:4, v/v) and eluting with DMF–chloroform (1:1, v/v). Solvent was removed under a stream of nitrogen and the adduct redissolved in methanol–water (3:7, v/v) and stored at −20°C. Sample purity was confirmed by HPLC using a $C_{18}$ column and gradient elution with acetonitrile–ammonium acetate buffer (50 m*M*, pH 4.0) with detection by UV absorbance at 315 nm (the absorbance maximum for PhIP) and 360 nm, the reported absorbance maximum for $N^2$-(2′-deoxyguanosin-8-yl)PhIP [8]. Analysis of the product by electrospray (ES) mass spectrometry with flow injection analysis confirmed structural identity and indicated an isotopic purity of >98%.

### 2.2.3. PhIP adducted calf thymus DNA

Calf thymus DNA (5 mg) was incubated with human liver microsomes, an NADPH regenerating system and [$^{14}$C]PhIP (50 μ*M*) in tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) buffer (50 m*M*, pH 7.4) containing 1 m*M* EDTA in a total volume of 10 ml at 37°C for 60 min. Periodically, acetic anhydride solution (a total of 2 ml of acetic anhydride–acetone, 1:1000, v/v) was added to the incubation mixture. DNA was precipitated overnight at −20°C by addition of 1/10 volume of 5 *M* sodium chloride and two volumes of absolute ethanol. After centrifugation and washing five times with cold (−20°C) absolute ethanol, the isolated pellet of DNA was dissolved in 150 m*M* sodium chloride–15 m*M* sodium citrate (pH 6.0) containing 0.1 m*M* EDTA. The covalent binding of PhIP to the DNA was estimated from the radioactivity of an aliquot of DNA solution, determined by liquid scintillation

counting. The concentration of DNA was determined spectrophotometrically at 260 nm, using an extinction coefficient of 20 mg/ml/cm.

### 2.3. Cell lines

#### 2.3.1. Cell culture

Metabolically competent human lymphoblastoid cells (h1A2v2 cells), genetically engineered to express human CYP1A2, were obtained from Gentest (Woburn, MA, USA) while human colon-derived HCT116 cells, which do not express CYP1A2, were obtained from ECACC (Porton, UK). The h1A2v2 cells were cultured according to the suppliers instructions in RPMI medium containing L-histidinol with 10% foetal bovine serum, at 37°C in a humidified atmosphere of 5% $CO_2$–95% air. The HCT116 cells were cultured similarly, but without L-histidinol.

#### 2.3.2. PhIP treatment of cells

h1A2v2 cells ($1.5 \cdot 10^6$) in exponential growth were incubated with PhIP (100 μ*M* in DMSO–water, 1:200, v/v) for 3 h and 24 h at 37°C. HCT116 cells were incubated with PhIP (100 μ*M*), human liver microsomes and an NADPH regenerating system for 1 h at 37°C.

#### 2.3.3. Extraction of DNA from cells

Cells, ($10^6$–$10^7$) were washed with phosphate-buffered saline. DNA was then extracted using the Nucleon BACC2/3 kit (Scotlab, Coatbridge, UK) according to the manufacturer's instructions.

### 2.4. Gas chromatography–mass spectrometry

#### 2.4.1. Hydrazinolysis

$N^2$-(2′-Deoxyguanosin-8-yl)PhIP (100 ng) or PhIP adducted DNA (100 μg) was added to 1,1-dimethyl hydrazine (50 μl) and flame sealed under nitrogen in a glass ampoule which was heated at 150°C for 18 h. The solution was then evaporated to dryness under nitrogen, the residue suspended in 1 *M* sodium carbonate solution (200 μl) and extracted with ethyl acetate. The organic extract was evaporated to dryness and derivatised for GC–MS analysis as described below.

#### 2.4.2. Alkaline hydrolysis

$N^2$-(2′-Deoxyguanosin-8-yl)PhIP standards (0, 0.2, 0.5, 2, 5 and 10 ng) or samples of PhIP adducted DNA (100 μg), to which had been added $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP (10 ng), were heated in 0.5 *M* sodium hydroxide (200 μl) at 100°C for 24 h in 1-ml Reacti-vials. Each hydrolysis mixture was extracted with ethyl acetate, the organic layer removed, evaporated under nitrogen and the residue derivatised for analysis by GC–MS.

#### 2.4.3. Derivatisation

A 5% solution of 3,5-bistrifluoromethylbenzyl bromide in acetonitrile (80 μl) and diisopropylethylamine (20 μl) were added. The reaction mixture was left at room temperature overnight and then excess reagents removed under nitrogen. The residue was dissolved in hydrochloric acid (0.1 *M*, 200 μl) and washed twice with hexane (750 μl). Sodium carbonate solution (0.5 *M*, 200 μl) was then added and the alkaline product extracted with ethyl acetate (2×750 μl). The organic solvent was removed under nitrogen and the residue reconstituted in dodecane (20 μl).

#### 2.4.4. Capillary column GC–electron-capture MS

A Finnigan MAT 4500 gas chromatograph quadrupole mass spectrometer (ThermoQuest, San Jose, CA, USA) was used. The gas chromatograph was equipped with a 15 m×0.25 mm I.D. DB5 J&W fused-silica capillary column, with helium as carrier gas at a head pressure of 70 kPa, and a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 270°C. The gas chromatograph oven temperature was held at 200°C for 1 min, then raised to 320°C at 20°C/min and held at this temperature for a further 1 min. Under these conditions, the retention times of the di(3,5-bistrifluoromethylbenzyl) derivatives of PhIP and [$^2H_3$]PhIP were 7.1 min The mass spectrometer was operated in the electron-capture negative ion mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 0.05 kPa and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was set to monitor negative ions of $m/z$ 449 and $m/z$ 452 and data acquisition and reduction were performed by an INCOS data system.

### 2.5. Liquid chromatography–mass spectrometry

#### 2.5.1. Enzymic digestion of DNA

Isolated DNA (100 μg) was digested according to the method described by Frandsen et al. [7]. Briefly, the DNA was dissolved in 200 μl buffer (30 m*M* sodium acetate, 1 m*M* zinc sulfate, pH 5.3), and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP (10 ng) added. The mixture was incubated with nuclease P1 (54 U/ml) and alkaline phosphatase (10 U/ml) at 70°C for 2 h then with micrococcal nuclease (54 U/ml) and alkaline phosphatase (10 U/ml) at 70°C for 2 h to digest DNA to nucleosides. The digest was treated with an equal volume of acetonitrile, then centrifuged to remove protein and the supernatant analysed directly by LC–MS.

#### 2.5.2. Microbore LC positive ion electrospray MS

$N^2$-(2′-Deoxyguanosin-8-yl)PhIP standards {containing 0, 0.2, 0.5, 1, 2, 5, 7.5 and 10 ng of adducted nucleoside together with 10 ng $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP} or digested DNA samples were chromatographed on a HiChrom RPB base deactivated column (10 cm×2.1 mm I.D.) preceded by an RPB guard cartridge (1 cm×2.1 mm I.D.) and coupled to a Waters 616 HPLC pump. Elution was isocratic, using acetonitrile–water (2:3, v/v) containing 0.04% heptafluorobutyric acid as solvent, and at a flow-rate of 70 μl/min the retention times of $N^2$-(2′-deoxyguanosin-8-yl)PhIP and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP were 6.8 min. The column was connected to a VG Quattro II mass spectrometer by a fused-silica capillary and the mass spectrometer was operated in the positive ion electrospray mode with a source temperature of 70°C and a cone voltage of 80 V. The mass spectrometer was tuned to monitor positive ions of *m/z* 374 and *m/z* 490 [$N^2$-(2′-deoxyguanosin-8-yl)PhIP] and *m/z* 377 and *m/z* 493 {$N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP}. Data acquisition and handling were performed with MassLynx software.

## 3. Results

The synthesis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP from PhIP is summarised in Fig. 1. $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP was made by the same synthetic route using the isotopically labelled precursor [$^2H_3$]PhIP. The identity of 2-nitro-[$^2H_3$]PhIP was confirmed by positive ion electrospray mass spectrometry and co-chromatography on TLC and HPLC systems with commercial 2-nitro-PhIP. The yield of the oxidation reaction was typically 30%. The *N*-hydroxy and acetoxy derivatives were not isolated but were used immediately to generate the $N^2$-(2′-deoxyguanosin-8-yl)PhIP adducts. Yields of $N^2$-(2′-deoxyguanosin-8-yl)PhIP from nitro-PhIP and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP from 2-nitro-[$^2H_3$]PhIP were typically 25%.

The UV spectrum of the purified $N^2$-(2′-deoxyguanosin-8-yl)PhIP adduct was consistent with that reported by others [7,8] with absorbance maxima at 360 nm and 240 nm and a shoulder at 270 nm. At 360 nm, the extinction coefficient for the adduct was determined to be 33 300±2600 l/mol/cm, based on 16 repeat analyses from four separate syntheses. The identity of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was confirmed by positive ion electrospray mass spectrometry. As has been reported previously [15], the mass spectrum of this compound contained a protonated molecular ion at *m/z* 490 and a major fragment ion at *m/z* 374. The latter represents the loss of the ribose group with the back-transfer of a proton to the base residue. The positive ion electrospray mass spectrum of $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP contained the expected protonated molecular ion at *m/z* 493 and major fragment ion at *m/z* 377. Under the LC–MS conditions employed, a mixture of $N^2$-(2′-deoxyguanosin-8-yl)PhIP and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP co-eluted and gave a mass spectrum of characteristic doublet ions (Fig. 2) with the isotopically unlabelled and labelled protonated molecular ions and fragment ions showing the expected difference of three mass units.

PhIP reacts with 3,5-bistrifluoromethylbenzyl bromide to give a di(3,5-bistrifluoromethylbenzyl) (di-TFMB) derivative suitable for GC–MS analysis (Fig. 3). Under conditions of electron-capture ionisation, the negative ion mass spectrum of this fluorinated compound contains a single major fragment ion at *m/z* 449, corresponding to loss of a TFMB group from the molecular ion. Amounts of derivative equivalent to 1 pg of the parent amine can be detected when the mass spectrometer is operated in the selected-ion monitoring mode. This sensitivity of

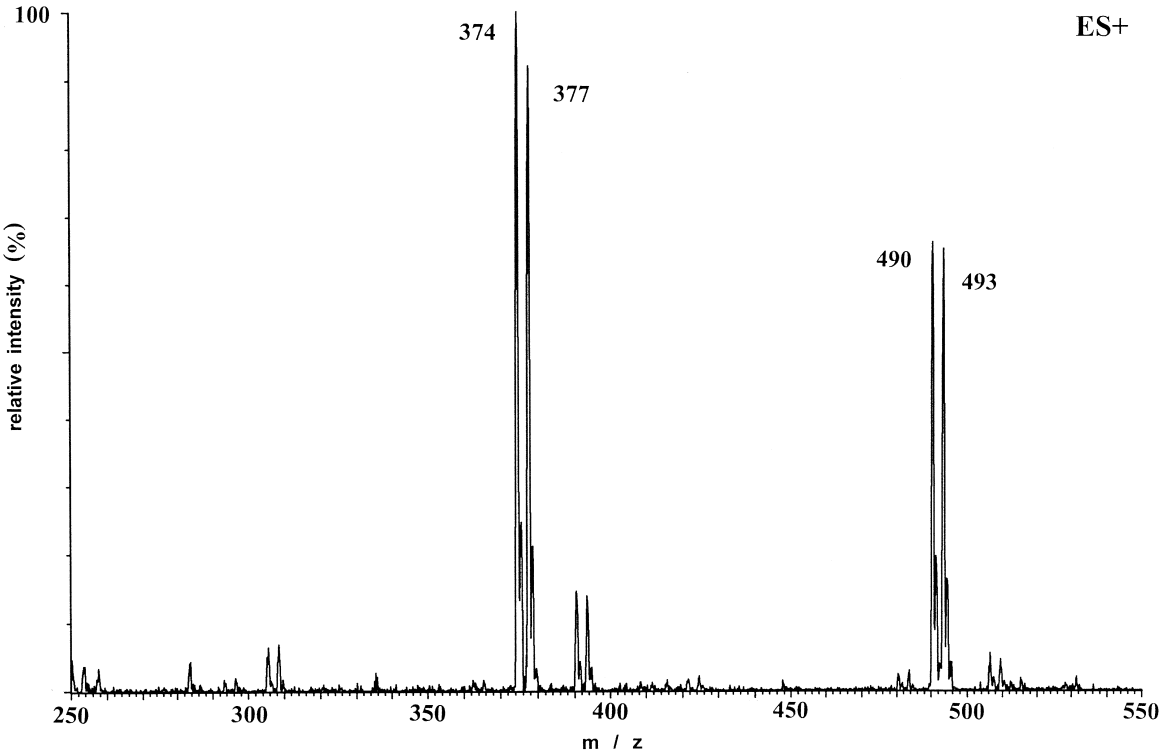


Fig. 2. The positive ion electrospray (ES+) mass spectrum of a 1:1 mixture of $N^2$-(2′-deoxyguanosin-8-yl)PhIP ($m/z$ 374, $m/z$ 490) and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP ($m/z$ 377, $m/z$ 493).

detection, together with the use of a deuterated analogue of PhIP as an internal standard, has already provided the basis of GC–MS assays for PhIP in food [14] and human urine [16].

The efficiencies of cleavage of PhIP from $N^2$-(2′-deoxyguanosin-8-yl)PhIP and PhIP adducted DNA by hydrazinolysis and alkaline hydrolysis were explored. The amount of PhIP released in each reaction

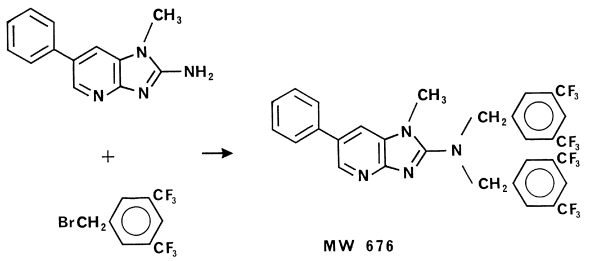


Fig. 3. Reaction of PhIP with 3,5-bistrifluoromethylbenzyl bromide to give a di(3,5-bistrifluoromethylbenzyl) (di-TFMB) derivative.

was determined using [$^2H_3$]PhIP as an internal standard in the GC–MS assay described above. While heating $N^2$-(2′-deoxyguanosin-8-yl)PhIP with 1,1-dimethylhydrazine at 150°C for 18 h released about 60% of PhIP bound to guanine, similar treatment of PhIP adducted DNA, in which the level of adduction was determined from radioactivity measurements, gave low (<25%) and variable yields of the parent amine. After optimising reaction conditions for the alkaline hydrolysis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP by varying molarity of sodium hydroxide, reaction time and temperature, a consistent yield of 60% was obtained. When PhIP adducted DNA was subject to alkaline hydrolysis, a similar yield of parent amine was obtained and this figure was found to be reproducible (57.9±0.7%, mean±SD, $n$=4). These results show that alkaline hydrolysis is much more efficient than hydrazinolysis at liberating PhIP from PhIP adducted DNA.

Using alkaline hydrolysis and GC–MS analysis, a standard curve was prepared with standards con-

taining $N^2$-(2′-deoxyguanosin-8-yl)PhIP (0–10 ng) and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP (10 ng). With selected-ion monitoring of ions of $m/z$ 449 (PhIP) and $m/z$ 452 ([$^2H_3$]PhIP), a six-point standard curve was obtained for the intensity ratio $I_{449}/I_{452}$ plotted against amount of $N^2$-(2′-deoxyguanosin-8-yl)PhIP added, and this was found to be linear ($y=0.0701x+0.010$, $r^2=0.998$). The presence of up to 500 μg DNA from control cells in the hydrolysis reactions did not alter the peak area ratios of the standards, indicating that no endogenous $N^2$-(2′-deoxyguanosin-8-yl)PhIP was present and that there was no chromatographic interference, but peak intensities were reduced by up to 50%. The limit of quantification (LOQ) of $N^2$-(2′-deoxyguanosin-8-yl)PhIP in the presence of DNA, defined as a signal-to-noise ratio for the PhIP derivative of 8:1, was 200 pg (189±18.4 pg, mean±SD, $n=4$; RSD 9.7%).

The GC–MS assay described above was used to analyse DNA extracted from h1A2v2 cells and HCT116 cells exposed to PhIP, and both cell lines showed that PhIP adduction had occurred. DNA extracted from h1A2v2 cells incubated with PhIP (100 μ*M*, 24 h at 37°C) showed a rate of PhIP adduct formation of 1.9 pmol/mg DNA/h (Fig. 4), while DNA from HCT116 cells incubated with PhIP (100 μ*M*, 1 h at 37°C) and a metabolic activation mixture showed a rate of PhIP adduct formation of 0.8 pmol/mg DNA/h. Equivalent samples of DNA were analysed unhydrolysed and no PhIP could be detected, indicating that the amine measured was bound covalently to the DNA. Incubation of HCT116 cells with PhIP in the absence of a metabolic activation system showed no adduction of PhIP to cellular DNA.

The possibility of using LC–MS for the direct analysis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was examined. Good chromatographic behaviour could be obtained on a reversed-phase, base deactivated, microbore column with isocratic elution, although connection to the mass spectrometer had to be made by a fused-silica capillary to eliminate peak tailing. With the mass spectrometer operated in the positive ion electrospray mode, a mass spectrum containing a protonated molecular ion at $m/z$ 490 and a major fragment ion at $m/z$ 374 was obtained. Selected-ion monitoring of the more intense ion at $m/z$ 374 allowed 50 pg of nucleoside to be detected with a signal-to-noise ratio of 4/1. The deuterated analogue of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was available for use as an internal standard and eight samples containing $N^2$-(2′-deoxyguanosin-8-yl)PhIP (0–10 ng)

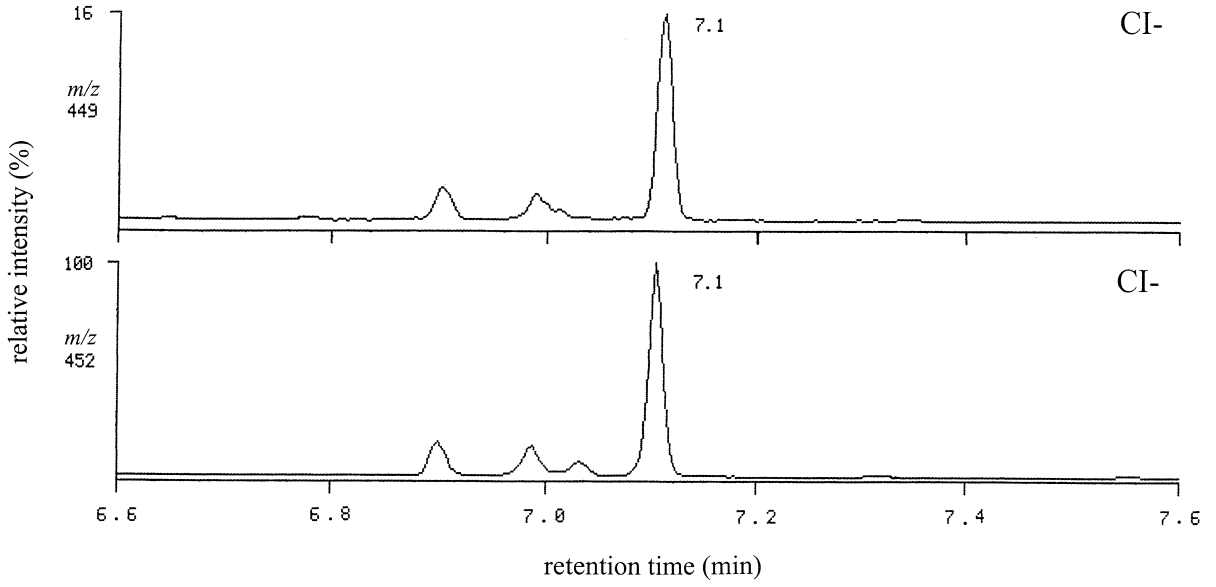


Fig. 4. GC–MS negative ion (CI−) chromatograms for the analysis of DNA from h1A2v2 cells exposed to PhIP ($m/z$ 449, PhIP diTFMB derivative, retention time 7.1 min; $m/z$ 452, [$^2H_3$]PhIP diTFMB derivative, retention time 7.1 min).

and $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP (10 ng) were prepared. With selected-ion monitoring of ions of $m/z$ 374 [$N^2$-(2′-deoxyguanosin-8-yl)PhIP] and $m/z$ 377 {$N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP}, the standard curve obtained for the intensity ratio $I_{374}/I_{377}$ plotted against amount of $N^2$-(2′-deoxyguanosin-8-yl)PhIP added was linear ($y=0.0885x+0.008$, $r^2=0.997$). Standards were added to samples of DNA from control cells that were then taken through the enzymic digestion procedure described above. The presence of up to 500 μg DNA did not alter the peak area ratios obtained, indicating that no endogenous $N^2$-(2′-deoxyguanosin-8-yl)PhIP was present and that there was no chromatographic interference, but peak intensities were reduced by up to 20%. Also a rapid decline in instrument sensitivity occurred after the analysis of approximately 20 DNA digests which required the removal and cleaning of the sample cone. The LOQ of $N^2$-(2′-deoxyguanosin-8-yl)PhIP in the presence of DNA, defined as a signal-to-noise ratio for the adducted nucleoside of 5:1, was 200 pg (212±37.4 pg, mean±SD, $n=4$; RSD 17.6%).

DNA was extracted from h1A2v2 cells that had been exposed to PhIP (100 μ*M*, 3 h at 37°C) and, following enzymic digestion, LC–MS analysis showed that $N^2$-(2′-deoxyguanosin-8-yl)PhIP was present (Fig. 5). The amount of nucleoside measured indicated a rate of PhIP adduct formation of 1.8 pmol/mg DNA/h.

## 4. Discussion

The GC–MS and LC–MS assays for $N^2$-(2′-deoxyguanosin-8-yl)PhIP described here are based on the use of a deuterated analogue of $N^2$-(2′-deoxyguanosin-8-yl)PhIP as an internal standard. The deuterated compound was made by the same route as the unlabelled adduct except that the synthesis

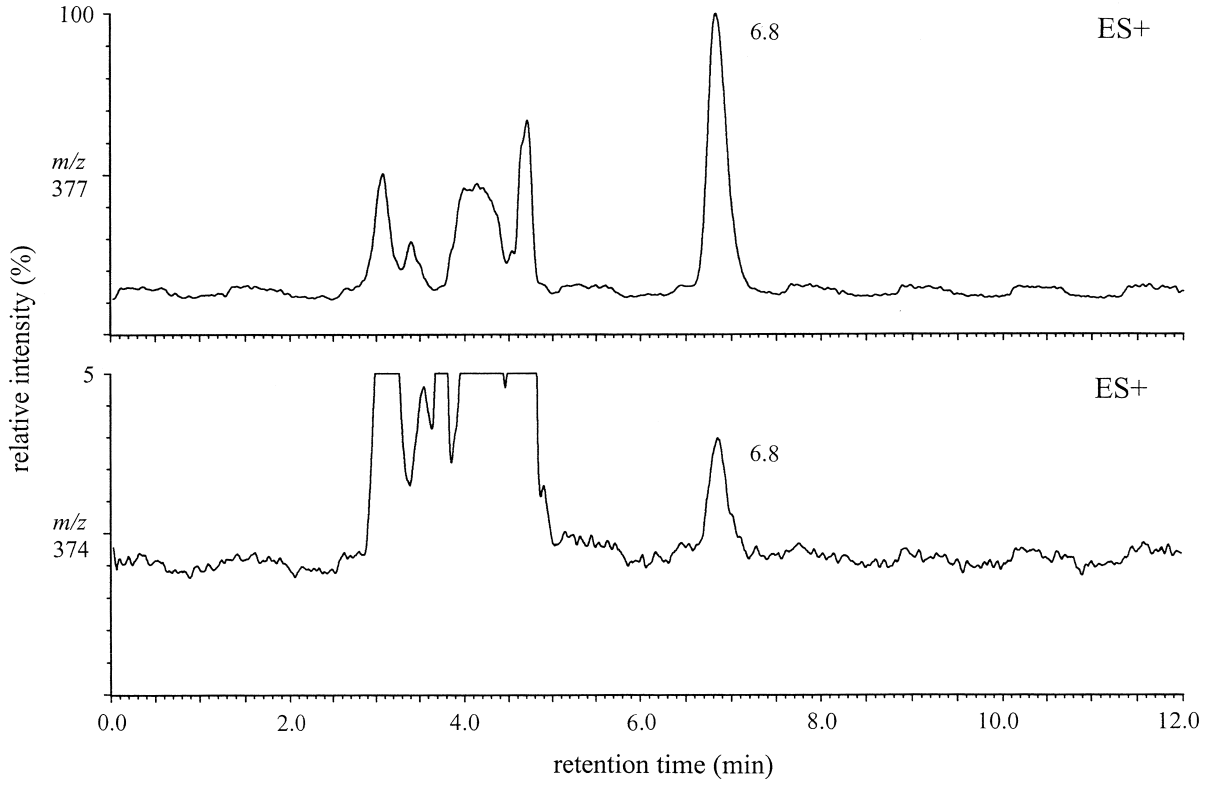


Fig. 5. LC–MS positive ion (ES+) chromatograms for the analysis of DNA from h1A2v2 cells exposed to PhIP {$m/z$ 374, $N^2$-(2′-deoxyguanosin-8-yl)PhIP, retention time 6.8 min; $m/z$ 377, $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP, retention time 6.8 min}.

proceeded from [$^2H_3$]PhIP (Fig. 1). The use of stable isotope labelled analogues for the quantitative analysis of analytes by mass spectrometry is widespread and preliminary experiments indicated that such an analogue of $N^2$-(2′-deoxyguanosin-8-yl)PhIP would be required here. Attempts to use [$^2H_3$]PhIP as an internal standard for the analysis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP by GC–MS were unsuccessful, even though the adduct was measured after chemical cleavage as the free amine. While estimates of the efficiency of the hydrazinolysis and alkaline hydrolysis procedures could be made this way, standard curves over a range of concentrations showed poor linearity due to variable, non-quantitative yields in the hydrazinolysis and alkaline hydrolysis reactions. Our findings are in contrast to those of Friesen and co-workers, who have reported the use of a pentadeuterated analogue of PhIP as an internal standard in a GC–MS assay using alkaline hydrolysis [10,11]. Direct measurement of $N^2$-(2′-deoxyguanosin-8-yl)PhIP by LC–MS using an external standard curve was similarly unsuccessful. While standard solutions of the adduct gave a reproducible response, analysis of the same samples which had been subjected to enzymic degradation in the presence of DNA showed a much poorer precision. The use of $N^2$-(2′-deoxyguanosin-8-yl)-[$^2H_3$]PhIP as an internal standard in the GC–MS and LC–MS assays overcame these problems and allowed linear standard curves to be constructed over the range 0–10 ng with coefficients of determination $>0.995$.

Comparison of hydrazinolysis and alkaline hydrolysis for the cleavage of PhIP from $N^2$-(2′-deoxyguanosin-8-yl)PhIP and PhIP adducted DNA showed that, while the two reactions were equally effective at releasing the amine from the synthetic adduct, hydrazinolysis was much less efficient at liberating PhIP from DNA. Hydrazinolysis has been used successfully in the measurement of acetylaminofluorene adducts of guanine, with the polyaromatic hydrocarbon being released and measured by GC–MS [17]. However, while hydrazinolysis of $N^2$-(2′-deoxyguanosin-8-yl)PhIP gave a 60% yield of the free amine, a much lower percentage was released from PhIP adducted DNA. Alkaline hydrolysis, being equally effective at releasing PhIP from $N^2$-(2′-deoxyguanosin-8-yl)PhIP and PhIP adducted DNA, was therefore the method of choice and, using this reaction and GC–MS analysis, covalent binding of PhIP to DNA extracted from h1A2v2 and HCT116 cells exposed to the amine was measured. Studies using inhibitors, inducers and specific antibodies have shown that the cytochrome P450 (CYP)1A enzyme family is primarily involved in the metabolic activation of PhIP, which is required prior to reaction with DNA [18,19]. Hence h1A2v2 cells, which have been genetically engineered to express human CYP1A2, showed adduction of PhIP to DNA (Fig. 4) while HCT116 cells, which do not express this enzyme, showed PhIP adduction only in the presence of an exogenous activation system (human liver microsomal fraction).

While previous work has shown that the principal site of adduction of PhIP to DNA is at the C-8 position of guanine [7–9], GC–MS analysis cannot distinguish between PhIP released from this adduct and PhIP released from other minor adducts that are also base labile. The LC–MS assay for $N^2$-(2′-deoxyguanosin-8-yl)PhIP in enzymic digests of DNA has a similar LOQ to the GC–MS assay, with the advantage that a specific adduct rather than a hydrolysis product is being measured. Analysis by LC–MS of DNA from h1A2v2 cells that had been exposed to PhIP showed the presence of this adduct (Fig. 5). Although these cells were incubated for a shorter period of time than the h1A2v2 cells analysed by GC–MS, the rate of formation of adduct calculated from the measured amount of $N^2$-(2′-deoxyguanosin-8-yl)PhIP was very similar to that obtained by GC–MS. The selected-ion monitoring traces obtained were free of any chromatographic interference and so the possibility of using multiple reaction monitoring (MRM) mass spectrometry, where greater specificity is obtained by the recording of product ions produced by collision induced dissociation, has not been explored. Rindgen et al. [15] have shown that MRM can be used to detect pg amounts of $N^2$-(2′-deoxyguanosin-8-yl)PhIP and further work assessing the relative sensitivities of the two techniques is currently in progress.

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